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# Cardiovascular disease risk associated with serum apolipoprotein B is modified by serum vitamin A

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ABSTRACT

*Background and aims:* Apolipoproteins B (apoB) and A1 (apoA1) are major protein constituents of low-density and high-density lipoproteins, respectively, and serum concentrations of these apolipoproteins are associated with risk of atherosclerosis. Vitamin A (VA) has been implicated in lipoprotein metabolism. We evaluated the associations of serum apoB, apoA1 and their ratio (apoBAR) with risk of incident acute myocardial infarction (AMI) and the possible modification by serum VA.
*Methods:* Risk associations were assessed by Cox regression, and presented as hazard ratios (HRs) per standard deviation (SD) increment in log-transformed values of the lipid parameters, among 4117 patients with suspected stable angina pectoris, located in Western Norway. Interactions with VA were evaluated by including interaction terms in the models. The multivariate model included age, sex, smoking, hypertension, number of stenotic coronary arteries, left ventricular ejection fraction, C-reactive protein, estimated glomerular filtration rate and statin treatment at discharge.
*Results:* Median (25th, 75th percentile) age of the 4117 patients (72% male) was 62 (55, 70) years. ApoB and apoA1 were higher among patients in the upper *versus* lower tertiles of VA. During a median of 4.6 (3.6, 5.7) years of follow-up, 8.2% of patients experienced an AMI. Overall, we observed no significant associations between lipid parameters and AMI after multivariate adjustment. However, apoB and apoBAR were associated with AMI among patients in the upper tertile of VA (HR per SD 1.35, (95% CI: 1.11 −1.65), and 1.42 (1.16−1.74), respectively, $p$ for interactions $\leq$0.003).
*Conclusions:* The associations of apoB and apoBAR with incident AMI were confined to patients with elevated VA.



## 1. Introduction

High systemic low-density lipoprotein (LDL) cholesterol and low high-density lipoprotein (HDL) cholesterol are associated with increased risk of atherosclerotic cardiovascular disease (CVD) [1]. The LDL particle is generated from very low density lipoproteins (VLDL) in the circulation and is involved in cholesterol transport from the liver to peripheral tissues, whereas the HDL particle is involved in reverse cholesterol transport from peripheral tissues to the liver [2,3]. The composition of the LDL particle includes one molecule of apolipoprotein B100 (apoB); thus, the number of apoB molecules equals the number of VLDL and VLDL-derived particles in the circulation [4]. Apolipoprotein A1 (apoA1) is the main constituent of HDL, and although each HDL particle may contain as

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much as five apoA1 molecules, systemic apoA1 levels may indicate HDL cholesterol concentrations [5]. However, apoA1 predicts CVD risk also independently of HDL cholesterol [6], and some studies show that apoB, apoA1 and their ratio (apoBAR) are considered more accurate predictors of CVD incidence than LDL and HDL cholesterol in untreated populations [7–10], as well as in patients receiving statin therapy [11,12].

Vitamin A (VA) designates a class of lipid-soluble nutrients collectively referred to as retinoids [13]. From the liver, VA is released as retinol bound to retinol-binding protein 4 (RBP4), which subsequently binds to transthyretin in plasma. In target tissues, retinol is converted to the bioactive retinoic acid (RA), which acts as a ligand for the transcription factors termed RA receptors. These receptors have possible target genes related to lipid and lipoprotein metabolism [14–16], and serum VA has been shown to be positively associated with serum triglycerides and total cholesterol in healthy adults [17,18]. Retinoid administration induces the enzyme glycine *N*-methyltransferase (GNMT) [19,20] in rodents, which may influence hepatic cholesterol trafficking with possible ramifications for VLDL composition [21]. Moreover, retinol is strongly associated with its transport protein in serum [22] and systemic RBP4 concentrations have been linked to cardiovascular outcomes in epidemiological studies [23,24]. Interestingly, both retinol-bound and retinol-free RBP4 promotes expression of scavenger receptors in macrophages [24]. These scavenger receptors bind to oxidized apoB-containing LDL particles, which in turn may lead to foam cell formation and progression of atherosclerosis [25]. Taken together, VA and lipoprotein metabolism seem to be interconnected. However, to the best of our knowledge, interactions between VA and apoB that influence risk of CVD have not been studied in humans.

The aim of this study was to assess the interaction of apoB, apoA1 and apoBAR with VA in relation to risk of AMI, in patients with suspected stable angina pectoris (SAP). More specifically, we conducted explorative interaction analyses to determine whether the risk relationships of apoB, apoA1 and apoBAR with incident AMI are modified by serum VA, and whether serum VA was an independent risk factor for incident AMI.

## 2. Patients and methods

Eligible subjects for this prospective study included 4164 patients undergoing elective coronary angiography for suspected SAP at Haukeland ($n = 3413$) and Stavanger ($n = 751$) University Hospitals, Norway, in the period 2000–2004. A total of 61.8% ($n = 2573$) of these patients participated in the Western Norway B-Vitamin Intervention Trial (WENBIT) (clinicaltrials.gov: NCT00354081) and received either 1) folic acid, vitamin $B_{12}$ and vitamin $B_6$, 2) folic acid and vitamin $B_{12}$, 3) vitamin $B_6$ or 4) placebo [26]. Subjects with missing values for serum apoB, apoA1 and/or VA ($n = 47$) were excluded, yielding a total of 4117 subjects available for analysis. All participants gave their written consent to participate. The study protocol was in accordance with the principles of the Declaration of Helsinki, and approved by the Regional Committee for Medical and Health Research Ethics, the Norwegian Medicines Agency, and the Norwegian Data Inspectorate.

Endpoint information was collected from the Western Norway Cardiovascular Registry [27] and the Norwegian Cause of Death Registry until December 31st, 2006. Incident AMI was classified according to the revised definition of myocardial infarction [28]. Non-fatal AMI occurring <24 h after baseline coronary angiography, percutaneous coronary intervention (PCI), or coronary artery bypass graft surgery (CABG) were excluded.

Information about medical history and medications at baseline and discharge from hospital were collected from each participant and verified by hospital records [26]. Trained personnel collected blood samples and registered blood pressure and anthropometric data. The majority (69.3%) of the population provided non-fasting samples. Hypertension was classified by preexisting diagnosis, and diabetics as participants previously diagnosed with diabetes (type 1 or 2) or having serum glucose ≥7.0 mmol/L or non-fasting serum glucose ≥11.1 mmol/L. Smoking status was determined by self-reported current smoking or having quit within the last four weeks, and verified by plasma cotinine ≥85 nmol/L measured as previously described [29]. The severity of coronary artery disease (CAD) was angiographically verified and scored between 0 and 3 according to the number of stenotic epicardial vessels with >50% lumen reduction. Fasting was defined as no ingested foods or beverages >6 h prior to blood sampling. Estimated glomerular filtration rate (eGFR) was determined according to the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula [30]. Dietary information was obtained from 2484 participants who completed a 169-item semi-quantitative food frequency questionnaire (FFQ) handed out on the day of inclusion and returned by mail or at the 1-month follow-up visit. The FFQ was developed at the Department of Nutrition, University of Oslo, Norway and was previously validated for several nutrients [31–34].

Serum blood samples were collected at baseline, and angiography was performed. Serum samples were stored at −80 °C until biochemical analyses. Serum concentrations of apoB and apoA1 were measured and determined by the Roche Hitachi 912 and 917 systems (Roche Diagnostics, GmbH, Mannheim, Germany), respectively. VA was measured as serum *all-trans*-retinol at BEVITAL AS (www.bevital.no) using liquid chromatography-tandem mass spectrometry as described previously [35]. Serum concentrations of C-reactive protein (CRP) were determined by an ultrasensitive immunoassay (N Latex CRP Mono, Behring Diagnostics, Marburg, Germany).

Continuous and categorical variables are presented as median (25th, 75th percentile) and percentage (%), respectively. Trends according to VA tertiles for continuous and categorical variables were assessed with unadjusted median linear or logistic regression. Skewed variables were log-transformed before analyses. Partial correlations for log-transformed VA with apoB, apoA1 and apoBAR were assessed with Pearson's correlation coefficient ($r$) and adjusted for age, sex, smoking, left ventricular ejection fraction and kidney function. Hazard ratios (HRs) per one standard deviation (SD) increment in log-transformed apoB, apoA1 and apoBAR, along with their respective 95% confidence intervals (CI), were calculated using Cox proportional hazards models. Two models were created: Model 1 was adjusted for age and sex, whereas model 2 was adjusted for age, sex, smoking, hypertension, number of stenotic vessels at coronary angiography, left ventricular ejection fraction, CRP, eGFR and statin prescription at discharge from hospital. Adjustment for diabetes, fasting status, and triglycerides did not alter the results materially and were thus not included in the final models. Potential effect modifications by VA were explored *post hoc* by adding interaction terms for apoB, apoA1 or apoBAR with VA (continuous), along with the separate variables in a multivariate model identical to Model 2. To determine whether the B-vitamin intervention affected the results, we carried out sensitivity analyses stratified by B-vitamin intervention in WENBIT. We considered $p$-values <0.05 significant. In order to adjust for the false discovery rate that may increase with multiple testing, the Benjamini-Hochberg procedure was applied [36]. The corrected significant $p$-value was set to <0.04.

Statistical analyses were carried out using the “base” and “survival” packages for R 3.0.2 (the R Foundation for Statistical Computing, Vienna, Austria).

## 3. Results

Baseline characteristics of the total study population are presented in Table 1. The population included 72% of men and 28% of women with a median (25th, 75th percentile) age of 62.0 (55, 70) years.

The baseline characteristics according to serum VA concentrations are shown in Supplemental Table 1. Median (25th, 75th percentile) circulating concentrations of VA were 2.30 (2.10, 2.45), 2.82 (2.69, 2.95) and 3.53 (3.29, 3.90) μmol/L in the 1st to 3rd VA tertiles, respectively. Participants with higher VA were more likely to have hypertension, and higher serum concentrations of triglycerides, apoB, apoA1 and total cholesterol, as well as lower CRP and eGFR. Moreover, patients in the upper VA tertile had less extensive CAD at coronary angiography. There were no trends according to statin, aspirin or beta-blocker prescription at baseline or discharge across VA tertiles. Finally, no trends were observed for dietary VA intake across tertiles of serum VA concentrations (data not shown).

The significant associations for VA with apoB and apoA1 at baseline were still present after multivariate adjustment for age, sex, smoking, left ventricular ejection fraction and eGFR ($r = 0.12$ and 0.19 respectively, both $p < 0.01$).

During median (25th, 75th percentile) 4.6 (3.6, 5.7) years of follow-up, 338 patients (8.2%) suffered an AMI. The risk associations with apoB, apoA1 and apoBAR are presented in Table 2. Overall, a trend of increased risk was found for apoB and apoBAR, whereas apoA1 was associated with a decreased risk of AMI in Model 1. These effects were attenuated by multivariate adjustment in Model 2. Moreover, no significant associations were found for VA and AMI in Model 1 (HR per SD: 1.10, 95% CI: 0.96–1.21, $p = 0.11$) or in Model 2 after multivariate adjustment (HR per SD: 1.07, 95% CI: 0.91–1.23, $p = 0.28$).

We observed a significant interaction between apoB and VA for AMI ($p$ for interaction = 0.003), whereas the interaction between apoA1 and VA was of borderline statistical significance ($p$ for interaction = 0.064). We also observed an interaction between apoBAR and VA in relation to AMI risk ($p$ for interaction<0.001). The interactions between lipid parameters and VA in relation to AMI risk were further explored by stratification according to the VA tertiles as presented in Table 2, and the same statistical models were used as for the total population. In Model 1, serum apoB concentrations and apoBAR were associated with increased risk of AMI only in the upper tertile of VA ($p < 0.001$), which remained significant in Model 2 ($p < 0.002$). Furthermore, apoA1 was inversely associated with AMI in Model 1 in the upper VA tertile ($p = 0.003$). This effect was slightly attenuated in Model 2 after inclusion of left ventricular ejection fraction in the multivariate model ($p = 0.152$).

In general, we observed similar results as in the total population among patients who received B-vitamin treatment in WENBIT (data not shown). However, in patients who received placebo or did not participate in WENBIT ($n = 2237$, No. of events = 181), we observed a trend for an interaction for apoB and VA in relation to AMI ($p$ for interaction = 0.062) whereas the interaction of apoA1 and apoBAR with VA in relation to AMI was significant ($p$ for interaction<0.001), as presented in Supplemental Table 2. After stratification, we found an inverse association between apoA1 and AMI in the upper VA tertile (HR per SD: 0.61, 95% CI: 0.47–0.80, $p < 0.001$) in the multivariate model. Finally, apoBAR was positively associated with AMI in the upper VA tertile (HR per SD: 1.71, 95% CI: 1.30–2.26, $p < 0.001$) in the multivariate model.

**Table 1**
Baseline characteristics of the participants.[a]

| | Total population |
|---|---|
| | $n = 4117$ |
| Age | 62 (55, 70) |
| Male sex, % | 72.0 |
| Hypertension, % | 46.8 |
| Diabetes, % | 11.0 |
| Current smoker, % | 31.6 |
| Previous CVD, % | |
| Previous AMI | 40.3 |
| Previous CBVD | 6.9 |
| Previous PVD | 9.0 |
| Previous CAD | 30.5 |
| Previous CABG | 11.5 |
| Previous PCI | 19.2 |
| Serum lipids | |
| LDL, mmol/L | 2.90 (2.40, 3.70) |
| HDL, mmol/L | 1.20 (1.00, 1.50) |
| TG, mmol/L | 1.50 (1.08, 2.14) |
| ApoA1, g/L | 1.30 (1.13, 1.48) |
| ApoB, g/L | 0.87 (0.73, 1.04) |
| ApoB/ApoA1 | 0.67 (0.54, 0.84) |
| Total cholesterol, mmol/L | 4.90 (4.30, 5.70) |
| Vitamin A, μmol/L | 2.82 (2.45, 3.29) |
| CRP, mg/mL | 1.78 (0.87, 3.67) |
| eGFR, mL/min per 1.73 $m^2$ | 91 (78, 99) |
| Extent of CAD, % | |
| No significant stenosis | 25.2 |
| One-vessel disease | 23.1 |
| Two-vessel disease | 22.3 |
| Three-vessel disease | 29.4 |
| LVEF <50 | 9.9 |
| Medication at baseline, % | |
| Statins | 72.4 |
| Aspirin | 80.3 |
| Beta-blocker | 73.4 |
| Medication at discharge, % | |
| Statins | 80.0 |
| Aspirin | 81.5 |
| Beta-blocker | 72.5 |

AMI, acute myocardial infarction; apo, apolipoproteins; apoBAR, apolipoproteinB/A1-ratio; CAD, coronary artery disease; CABG, coronary artery bypass graft surgery; CBVD, cerebrovascular disease; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate, LVEF, left ventricular ejection fraction; PVD, peripheral vascular disease; PCI, percutaneous coronary intervention; TG, triglycerides; VA, vitamin A.

[a] Categorical variables are presented as % and continuous variables as medians (25th, 75th percentile).

## 4. Discussion

In this large prospective study among 4117 patients with SAP, we show for the first time that the risk relations of lipid-related parameters were modified by serum concentrations of VA. Initially, we did not observe an association for apoB, apoA1 and apoBAR with AMI following multivariate adjustment. However, interaction analyses revealed that serum concentrations of apoB and apoBAR were associated with increased risk of AMI in patients with elevated VA only. We also showed, in a sensitivity analysis, that the interaction between apoA1 and VA reached statistical significance in participants not receiving B-vitamin treatment.

There are several possible biological mechanisms that may explain the statistical interaction we observed in this study. The retinol transport protein, RBP4, has been associated with risk of CVD, and a recently published study showed that both retinol-bound and retinol-free RBP4 promoted CD36 expression and cholesterol uptake in macrophages [24]. Moreover, one *in vitro* study of cells incubated with *all-trans* RA demonstrated a rapid induction of CD36 on both the mRNA and protein level [37]. This scavenger receptor is present on the surface of macrophages and binds to apoB-containing oxidized LDL particles, which are implicated in foam cell formation, platelet activation, inflammation and

**Table 2**
Associations between apolipoproteins B and A1 and their ratio with acute myocardial infarction according to tertiles of vitamin A.

| | Total population | | Vitamin A tertiles | | | | | | *p* for interaction |
|---|---|---|---|---|---|---|---|---|---|
| | HR per SD (95% CI) | *p* | <2.58 μmol/L | | 2.58–3.09 μmol/L | | >3.10 μmol/L | | |
| | | | HR per SD (95% CI) | *p* | HR per SD (95% CI) | *p* | HR per SD (95% CI) | *p* | |
| ApoB | | | | | | | | | 0.003 |
| Model 1[a] | 1.11 (1.00–1.24) | 0.058 | 0.92 (0.78–1.10) | 0.356 | 1.07 (0.88–1.30) | 0.489 | 1.42 (1.17–1.73) | <0.001 | |
| Model 2[b] | 1.09 (0.98–1.22) | 0.115 | 0.91 (0.76–1.09) | 0.287 | 1.08 (0.88–1.32) | 0.458 | 1.35 (1.11–1.65) | 0.002 | |
| ApoA1 | | | | | | | | | 0.064 |
| Model 1 | 0.85 (0.76–0.94) | 0.003 | 0.99 (0.82–1.19) | 0.904 | 0.75 (0.61–0.93) | 0.007 | 0.76 (0.63–0.91) | 0.003 | |
| Model 2 | 0.95 (0.85–1.06) | 0.359 | 1.10 (0.91–1.34) | 0.313 | 0.83 (0.66–1.03) | 0.084 | 0.87 (0.71–1.05) | 0.152 | |
| ApoB/A1 | | | | | | | | | <0.001 |
| Model 1 | 1.21 (1.08–1.34) | <0.001 | 0.94 (0.78–1.13) | <0.001 | 1.22 (1.01–1.47) | 0.040 | 1.60 (1.31–1.95) | <0.001 | |
| Model 2 | 1.11 (0.99–1.24) | 0.070 | 0.87 (0.73–1.05) | 0.135 | 1.17 (0.96–1.43) | 0.112 | 1.42 (1.16–1.74) | <0.001 | |

apoB, apolipoprotein B; apoA1, apolipoprotein A1; apoB/A1, ratio of apolipoprotein B to A1; HR per SD, hazard ratio per standard deviation increase.
[a] Cox proportional hazards model adjusted for age and sex.
[b] Cox proportional hazards model adjusted for age, sex, smoking, C-reactive protein, number of stenotic vessels, left ventricular ejection fraction, hypertension, statin use at discharge from hospital and estimated glomerular filtration rate.

ultimately the progression of atherosclerosis [25]. Finally, a study in vascular smooth muscle cells demonstrated a ~7-fold induction of the lectin-like oxidized LDL receptor by *all-trans* RA [38], which, upon binding of oxidized LDL, may promote endothelial dysfunction, foam cell formation and vascular smooth muscle cell apoptosis in atherosclerotic lesions [25].

The administration of *all-trans*-RA has been associated with enhanced expression of glycine-*N*-methyltransferase (GNMT) in rodents [19,20]. This enzyme has recently been implicated in cholesterol metabolism, as GNMT knock-out mice exhibit hepatic lipid accumulation, suggesting a possible role of GNMT in hepatic VLDL export [39]. In cell cultures, GNMT has been associated with Niemann Pick-C2, a protein essential for cholesterol transport in the liver [21], with potential ramifications for VLDL composition and export. Thus, VA activity in the liver may be important for the synthesis, packaging and secretion of VLDL from the liver to the peripheral tissues, via alterations in GNMT. However, preclinical studies have also suggested that GNMT inhibition in atherosclerotic plaques may impair reverse cholesterol transport (RCT) [39], a process by which apoA1-containing HDL particles play a crucial role. Notably, we observed a trend towards an inverse relationship between apoA1 and incident AMI among patients with high VA, particularly in those that did not receive B-vitamin treatment, including folate, which is a potent inhibitor of GNMT [40].

In this study, we present a significant interaction of apoB and apoBAR with circulating concentrations of VA in relation to AMI. As VA is an abundant nutrient in several affluent societies, a high intake may promote adverse effects in combination with elevated serum apoB and apoBAR. However, VA intake did not correlate with circulating concentrations and did not differ across VA tertiles in our population, and is unlikely to influence the observed interaction. We observed effect modifications of apoB and apoBAR by VA in patients with SAP. If confirmed in other populations, determination of VA in combination with apoB, apoA1 and apoBAR may become useful in CVD risk assessment. Additionally, it may be useful to reanalyze data from larger existing studies to assess whether the CVD risk associated with apoB, apoA1 and apoBAR is modified by VA. Finally, the observed interaction may clarify the observed increased CVD mortality in a population receiving vitamin A supplements [41].

The major strength of this study is its large and well-characterized population with long-term follow-up. Moreover, as the median circulating concentrations of VA in the total population and across VA tertiles were generally higher as compared with other studies [17,42], this population is well-suited for the study of high concentrations of VA and the possible interaction with traditional risk markers.

Our study has several limitations. The majority of blood samples were drawn from non-fasting subjects, which may affect circulating concentrations of serum lipids. However, when we adjusted for fasting status and serum triglycerides in the multivariate model, the effect sizes and interactions remained unchanged. The majority of the population (>80%) received apoB-lowering statin treatment, and the observed effect sizes for apoB and apoBAR may be biased in the total population, as well as the VA tertiles, and not generalizable to untreated populations. However, apoB is associated with CVD also in statin-treated populations [43] and our results may thus hold merit for patients undergoing lipid-lowering therapy. Additionally, in our sensitivity analysis, the B-vitamin treatment seems to mask the interaction of apoA1 with VA, and we cannot exclude further bias introduced by the trial intervention. The interpretation of these results are further complicated by the finding that VA levels in blood seem to be high when levels in tissues are low [44], and the fact that we did not measure RA, which is the major bioactive retinoid. It should be noted that little is known about the regulation of serum VA concentrations, as it is generally thought to be unaffected by dietary intake [45]. However, one recently published study showed that serum VA was slightly higher in supplement users than in non-users [46]. Thus, we cannot exclude the possible influence of unreported long-term high-dose supplementation on circulating concentrations of VA among our participants. Moreover, there may also be other unmeasured and unknown factors that affect serum concentrations of VA that have not been accounted for.

In conclusion, our results suggest that the risk of incident AMI associated with apoB and apoBAR is modified by VA whereas the interaction between apoA1 and VA was only present in patients not receiving B-vitamins in the original trial. Reanalysis of data from existing trials with VA supplements would be useful to determine whether potential risk associated with such treatment is via modification of circulating apolipoprotein concentrations. Finally, the possible mechanisms by which VA potentially increases the atherogeneity of apoB should be elucidated in experimental models.

## Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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## Author contributions

OKN conceived and designed the study; OKN, GFTS, ERP and PMU conducted the research; TO and KJV conducted the statistical analyses; ØM and PMU were responsible for measuring vitamin A in serum; TO, KJV, GFTS, ERP, GST, RB, CAD, PMU, HR and OKN interpreted the results; TO, KJV, GFTS, ERP, GST, RB, CAD, PMU, ØM, HR and OKN wrote, critically revised and approved the final version of the manuscript.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2017.07.020.

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